Chapter 8
Notch Signaling in T-Cell Acute Lymphoblastic Leukemia and Other Hematologic Malignancies

Catherine Hoofd, Vincenzo Giambra, and Andrew P. Weng

Abstract Notch is a highly conserved signaling pathway that is crucial for development and homeostasis of many normal tissues and cell types. Deregulated Notch signaling is associated with human disease in several different tissue contexts but is perhaps best characterized in T-cell acute lymphoblastic leukemia/lymphoma (T-ALL). Activating mutations in the *NOTCH1* gene and other elements of the Notch signaling pathway such as *FBW7* result in increased Notch signaling intensity and/or duration and are acquired spontaneously at high frequency in primary human T-ALL and in experimentally derived mouse models of T-ALL. As well, enforced expression of activated NOTCH1 in normal hematopoietic progenitors promotes cellular transformation and leads to development of T-ALL-like disease in mice. Recent work has highlighted a role for the Notch pathway in other hematologic malignancies as well. While gain-of-function mutations in NOTCH receptors occur frequently in mature B-cell malignancies such as chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), and splenic marginal zone lymphoma (SMZL), activation of the Notch pathway can also block tumor progression in myeloid malignancies, highlighting its highly versatile and context-dependent nature. In this chapter, we summarize the most recent findings regarding the pathogenic role of Notch signaling in various hematologic malignancies and current strategies to inhibit it therapeutically.

Keywords Notch · Leukemia · Lymphoma · T-ALL · Signal transduction · Gene mutation · γ-secretase inhibitor · Mouse model

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L. Miele, S. Artavanis-Tsakonas (eds.), *Targeting Notch in Cancer*,
https://doi.org/10.1007/978-1-4939-8859-4_8

8.1 Notch Signaling Pathway

8.1.1 Notch Receptors and Ligands

The Notch signaling pathway is highly evolutionarily conserved and provides for communication between neighboring cells that is important for normal tissue development and homeostasis. In mammals, there are four Notch receptors (NOTCH1–4) and multiple ligands of the Delta/Serrate/Lag-1 (DSL) family including Delta-like (DLL)-1, DLL-3, and DLL-4 and Jagged (JAG)1 and JAG2. All four Notch receptors are single-pass transmembrane proteins that include multimerized epidermal growth factor (EGF) repeats within the extracellular domain which mediate interaction with DSL ligands. Glycosyltransferase homologs of the Fringe family including Lunatic, Manic, and Radical Fringe [1, 2] can modify specific EGF repeats that provide for ligand selectivity [3–5].

Notch receptors are initially translated as a single polypeptide but are cleaved at the S1 site by a furin-like protease in the trans-Golgi [6] into two subunits that noncovalently reassociate before trafficking to the cell surface. The extracellular domain consists of EGF repeats that mediate interactions with ligand, three tandem Lin-12/Notch repeats (LNR), and the N-terminal portion of the heterodimerization domain (HD^N). HD^N associates with its partner C-terminal HD domain (HD^C) which resides at the N-terminus of the transmembrane subunit and holds the two subunits together [7, 8]. The LNR portion "drapes" over the HD domain to shield it from cleavage by intramembrane proteases [9, 10] (Fig. 8.1).

Ligand binding and subsequent bilateral endocytosis (ligand endocytosis by the signal-"sending" cell and receptor endocytosis by the signal-"receiving" cell) is thought to exert a physical pull that displaces the LNR domain, thus exposing the S2 site within the HD^C domain to proteolytic cleavage by an intramembrane ADAM metalloprotease [11–13]. This reveals yet another proteolytic cleavage site S3 near the inner leaflet of the plasma membrane that is acted upon by γ-secretase, a multisubunit intramembrane protease complex consisting of presenilin 1 or 2, PEN-2, APH-1, and nicastrin. Cleavage at the S3 site releases the intracellular domain (ICN) from its membrane tether, and ICN is then free to translocate to the nucleus by virtue of its two nuclear localization signals (Fig. 8.2).

ICN itself contains an Rbp-associated molecule (RAM) domain which mediates association with the DNA-binding factor CSL (CBF1, Suppressor of Hairless, Lag-2; also known as RBPJ), an ankyrin repeat domain (ANK) which mediates protein-protein interactions, a transactivation domain (TAD), and negative regulatory proline/glutamic acid/serine/threonine-rich (PEST) domain at the C-terminus [3, 14] (Fig. 8.1). CSL can interact with various cofactors to build either repressor complexes containing histone deacetylases such as SMRT/NCoR [15, 16] or activator complexes with ICN and Mastermind-like (MAML) proteins which recruit chromatin-modifier proteins such as histone acetyltransferases p300 and pCAF [17, 18]. CSL/ICN/MAML transcriptional complexes also recruit kinases such as CDK8

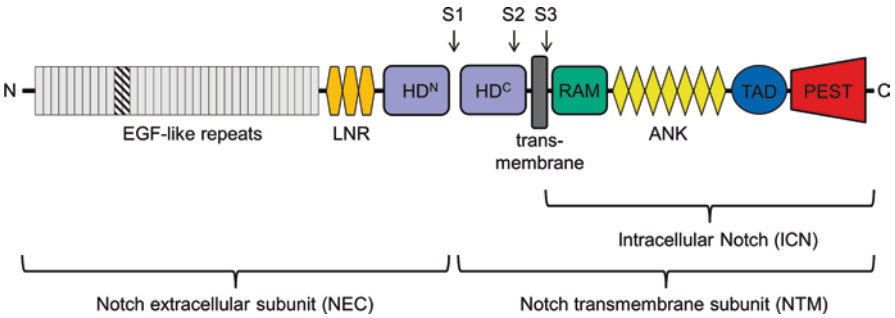

Fig. 8.1 Structure of human NOTCH1. *NOTCH1* is translated as a single polypeptide that is cleaved by a furin-like protease at the S1 site, yielding two subunits that non-covalently reassociate prior to trafficking to the cell surface. The extracellular subunit (NEC) includes epidermal growth factor (EGF) repeats 11–12 (cross-hatched bars) involved in ligand binding, three tandem Lin-12/Notch repeats (LNR), and the N-terminal portion of the heterodimerization domain (HD^N). The transmembrane subunit (NTM) includes the C-terminal portion of the HD (HD^C), a transmembrane domain, the Rbp-associated molecule (RAM) domain which binds CSL, ankyrin repeats (ANK), a transactivation domain (TAD), and a C-terminal negative regulatory proline/glutamic acid/serine/threonine-rich (PEST) domain. Ligand binding produces an allosteric structural change affecting the HD domain, exposing the S2 site to proteolytic cleavage by an ADAM metalloprotease. This reveals the S3 site, which is cleaved in turn by γ-secretase, releasing intracellular Notch (ICN) from the plasma membrane

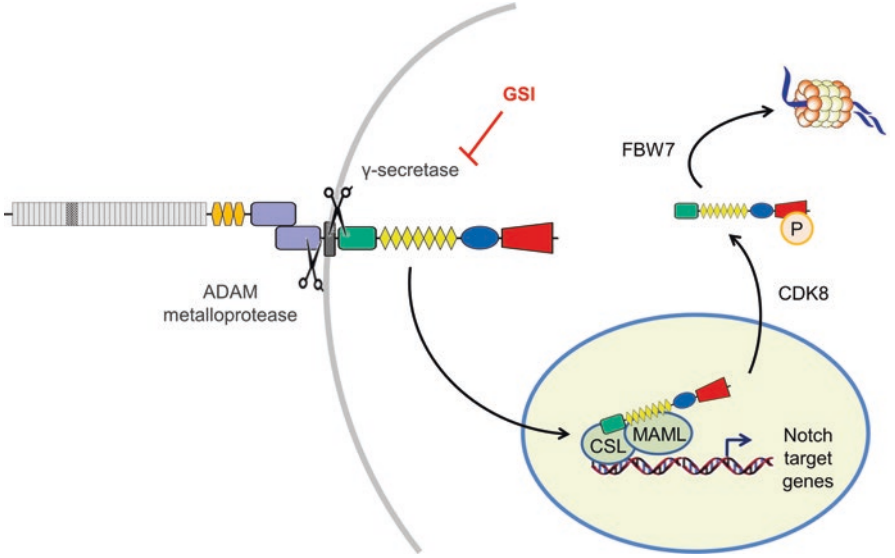

Fig. 8.2 The canonical Notch signaling pathway. Ligand binding induces sequential proteolytic cleavages by ADAM metalloprotease and γ-secretase, releasing ICN from the membrane. ICN then translocates to the nucleus where it forms a transcriptional complex with the DNA-binding factor CBF1/Suppressor of hairless/Lag-1 (CSL; also known as RBPJ) and the co-activator Mastermind-like (MAML) to drive expression of downstream target genes. Signaling is terminated by phosphorylation, ubiquitination, and ultimately proteasomal degradation of ICN. GSI γ-secretase inhibitor, CDK8 cyclin-dependent kinase 8, FBW7 F-box and WD repeat domain-containing 7

that phosphorylate ICN within the PEST domain, targeting it for ubiquitination by FBW7 and subsequent proteasomal degradation, thus terminating the signaling event [19, 20] (Fig. 8.2).

Notch transcriptional complexes can function either as monomers or as dimers when bound to paired head-to-head CSL-binding sites [21, 22]. In particular the ICN1 residue R1985 is involved in the interaction of ANK domains of ICN1 molecule [23]. Of note, specific mutations (e.g., R1985A) abrogate the formation of Notch dimeric complex and prevent the induction of T-ALL in mice [24], suggesting that the Notch target genes involved in leukemogenic transformation are modulated via Notch dimerization on paired sites.

8.1.2 Notch Target Genes

One recurring theme in Notch signaling is that the precise outcome of signaling is highly dependent on cellular and developmental context. Accordingly, Notch signaling may alternatively promote self-renewal or differentiation, proliferation or cell cycle arrest, and survival or apoptosis. These disparate cellular outcomes are presumably mediated in part by different complements of target genes activated, directly or indirectly, downstream of Notch. For instance, *CD25* [25], *PTCRA* [26], and *GATA3* [27] represent cell type-specific Notch targets and manifest developmental stage-specific cellular outcomes [28]. Despite this, some target genes are consistently downstream of Notch in multiple tissue contexts. Most notably, members of the Hairy/Enhancer of Split (*HES*) gene family are induced directly by Notch in several different tissue contexts besides T-ALL such as neural cells where they control cell fate and muscle and intestinal cells where they guide normal development. *HES* genes encode basic helix-loop-helix (bHLH) DNA-binding proteins that function as transcriptional repressors by recruiting corepressors of the transducing-like enhancer (TLE) family [29, 30]. Other Notch targets that are relatively conserved across different tissue contexts include *DTX1*, an ubiquitin ligase that can regulate Notch trafficking at the membrane [31], and *NRARP*, which can feedback to negatively regulate Notch signaling [32–34].

Another Notch target of particular interest is *MYC* due to its important role in human cancer. Indeed, NOTCH1 has been shown to induce *MYC* expression directly in multiple cancer types including T-ALL [35–38] and breast cancer [39]. Interestingly, although initial work focused on CSL-binding sites residing near the *MYC* promoter, subsequent studies revealed a critical Notch-dependent distal enhancer located ~1.5 megabases downstream of the human *MYC* gene that is broadly conserved between mammals, birds, and reptiles [35, 40, 41]. This enhancer was shown to loop back to the *MYC* promoter by chromatin conformation capture (3C) assay; however, the topology was stable despite γ-secretase inhibitor (GSI) treatment, implying that NOTCH1 occupancy is not required to maintain the chromatin loop. Other cancer-relevant Notch targets include *CCND1* [42] and the tumor suppressor *CDKN1A* [43].

8.2 Notch and Early Hematopoiesis

Notch plays important roles throughout hematopoietic development. Notch1 signaling is required very early in embryonic hematopoiesis including during development of the first definitive hematopoietic stem cells (HSC) [44, 45]. Subsequent fetal HSC development has also been shown to be dependent on NOTCH1 through the use of transactivation domain (TAD) mutant mice [46]. Initial gain-of-function experiments showed that NOTCH1 supported expansion of adult HSC [47, 48]; however, subsequent loss-of-function experiments showed Notch signaling to be dispensable for adult HSC maintenance [49], suggesting HSC expansion may represent an artifact of supraphysiological levels of signaling.

8.3 Notch and T-Cells

8.3.1 NOTCH1 Signaling in Normal T-Cell Development

Perhaps the greatest amount of work has focused on the role of Notch signaling in normal T-cell development. Both gain- and loss-of-function experiments have highlighted that NOTCH1 critically directs lymphoid progenitors in a binary fate decision between B and T lineages. In particular, inducible deletion of *Notch1* or *Rbpj* in hematopoietic progenitors suppresses T-cell development, resulting in accumulation of ectopic B-cells in the thymus, whereas constitutively activated NOTCH1 promotes T-cell differentiation within the marrow and at the expense of B-cells [50–52]. While both DLL1 [53] and DLL4 ligands [54] are capable of supporting T-cell development in vitro, stromal cues guiding NOTCH1 activation during normal intrathymic T-cell development are provided by the ligand DLL4 as expressed on thymic epithelial cells [55, 56], whereas DLL1 has been shown to be dispensable for this process [57]. NOTCH1 signaling can also influence binary cell fate decisions at later stages of T-cell development including between αβ and γδ lineages [58] and between CD4 and CD8 [59, 60] or Th1 and Th2 differentiation [61].

8.3.2 NOTCH1 Signaling in T-Cell Acute Lymphoblastic Leukemia (T-ALL)

The first evidence of an oncogenic role for Notch was the discovery of balanced t(7;9) translocations involving the T-cell receptor β (TRB) locus on chromosome 7 and the *NOTCH1* gene on chromosome 9 in rare cases of human T-ALL by Jeff Sklar's group [62]. This translocation resulted in expression of 5' truncated *NOTCH1* transcripts in developing T-cells which encoded constitutively active forms of the receptor [62]. As well, retroviral insertional mutagenesis screens in mice have

reported that common proviral insertions occur near or within the extracellular negative regulatory region (NRR) of *Notch1* that presumably result in viral LTR-driven expression of similarly truncated forms of the receptor [63–66]. Subsequent work by Warren Pear and others demonstrated that enforced expression of similar N-terminally truncated forms of human NOTCH1 in mouse bone marrow-derived hematopoietic stem progenitor cells (HSPC) by retroviral transduction followed by transplantation into syngeneic recipients resulted in short-latency, high-penetrance T-ALL-like disease in mice [67–69]. The potency of activated NOTCH1 in producing T-ALL was also confirmed by distinct but similar experimental approaches including transgenic mice [70, 71] and transgenic zebrafish [72] and more recently by our own group, using human cord blood progenitors (Kusakabe et al., manuscript in preparation). Of note, less potently activated forms of NOTCH1 arrest T-cell development at the CD4+CD8+, or "double-positive" (DP) stage, but do not produce T-ALL disease, suggesting that increasing thresholds of NOTCH1 signaling are required for effects on T-cell development and transformation [73–75]. Importantly, inhibition of NOTCH1 in each of these settings results in reduced growth and/or apoptosis of T-ALL cells and can be accomplished either genetically with reagents like dominant-negative MAML1 (dnMAML1) [76] or pharmacologically with γ-secretase inhibitors (GSI) [76] or anti-NOTCH1 antibodies [77].

8.3.3 NOTCH1 Mutations in T-ALL

Despite work in mouse models, *NOTCH1* was generally regarded as a "boutique" oncogene in human T-ALL whose involvement was limited to those rare cases harboring the classic (7;9) chromosomal translocation. This view was revised following the discovery of point mutations and small indels in *NOTCH1* leading to gain-of-function in ~60% of human T-ALL by Jon Aster's group [78]. This discovery came as a result of screening human T-ALL cell lines for sensitivity to gamma-secretase inhibition as we had recently shown this was effective against mouse T-ALL generated with activated NOTCH1 [76]. As well, our motivation to look within the HD domain for mutations was critically informed by structural studies in Steve Blacklow's lab that suggested it played an important role in maintaining structural integrity of the receptor and restraining its activation [8]. Others have subsequently confirmed these findings in larger and varied cohorts using targeted or whole genome/exome sequencing [79–88] (Table 8.1).

Activating mutations in *NOTCH1* are distributed predominantly within the two regions, the heterodimerization (HD) domain and the C-terminal PEST domain. HD mutations occur in 40–45% of human T-ALL cases and can be divided in two distinct structural classes [99]. The more common class I mutations consist of small deletions involving at most a few amino acids, short in-frame insertions, or single amino acid substitutions within exons 26 and 27 that encode N- and C-terminal halves of the HD domain, respectively. These class I alterations maintain the reading frame and destabilize or completely disrupt physical association between the

Table 8.1 Frequencies of *NOTCH* mutations in hematologic malignancies

Disease	NOTCH receptor (domain)	Frequency of mutation	References
T-ALL	NOTCH1 (HD)	40–45%	[78–88]
	NOTCH1 (PEST)	20–25%	
CLL	NOTCH1 (PEST)	10–30% (mostly delCT)	[89–93]
MCL	NOTCH1 (PEST)	5–10%	[94, 95]
	NOTCH2 (PEST)	5%	
SMZL	NOTCH1 (PEST)	5%	[96, 97]
	NOTCH2 (PEST)	20–25% (mostly delCT)	
DLBCL	NOTCH2 (PEST)	8%	[98]

two HD subunits, thereby reducing the threshold for ligand-mediated activation or spontaneously activating the receptor outright, respectively [99]. Conversely, class II mutations are rare and consist of tandem insertions of 12–15 amino acids which duplicate the S2 cleavage site in the C-terminal portion of the HD domain. The presumed mechanism for activation by class II mutations is informed by structural studies of the extracellular negative regulatory region (NRR), which would predict that the duplicated HD region places an extra S2 cleavage site beyond the protection of the NRR [9, 10]. Yet a third but again rare type of activating *NOTCH1* mutation, so-called juxtamembrane expansion (JME), introduces additional in-frame amino acids just external to the cell membrane and proximal to the HD domain which render the receptor more susceptible to S2 cleavage, possibly by destabilizing interaction of the otherwise intact NRR/HD complex with integral membrane proteins or allowing intramembrane proteases illegitimate access to the base of the receptor stalk [100].

Genetic alterations within the C-terminal PEST domain occur in 20–25% of T-ALL cases and consist of nonsense and frameshift mutations that lead to premature stop codons [78]. These truncated polypeptides lack critical portions of the PEST domain required for ubiquitination and proteolytic turnover of intracellular NOTCH1 (ICN1) [101, 102]. The deleted region of the PEST domain consistently includes a highly conserved sequence, ^{2521}WSSSSP^{2526}, which contains important phosphorylation site(s) that are required for subsequent ubiquitination [103]. Other common deletions fall between the ^{2482}FLTPPSQ^{2488} and ^{2510}FLTPSPE^{2516} sequences, each of which are recognized by E3 ligase complexes containing the F-box protein FBW7 and are similar to the ^{55}LLPTPPLSP^{63} sequence present in MYC that regulates its proteolytic turnover [104]. The ultimate result of these alterations is reduced proteolytic turnover of ICN1 and prolonged duration of signaling following receptor activation.

It remains unresolved which kinases modulate activity and turnover of ICN1. It has been reported that the cyclin-dependent kinase 8 (CDK8) interacts physically with the Notch transcriptional complex (ICN1, Mastermind-like-1 (MAML1), and CSL) and phosphorylates specific serine residues on ICN1 including S2514, S2517, and S2539 [19]. These phosphorylation events are then thought to target ICN1 for subsequent ubiquitination and proteasomal degradation. Accordingly, mutations

affecting these residues could lead to reduced ICN1 turnover and prolonged duration of signaling that could contribute to T-ALL pathogenesis.

Akin to NOTCH1 PEST deletion, there is also biological selection in T-ALL for inactivating mutations in *FBW7*, the ubiquitin ligase that is responsible for targeting ICN1 for proteasomal degradation. Inactivating *FBW7* mutations occur in 10–20% of T-ALL and are mutually exclusive to *NOTCH1* PEST mutations [79, 101, 102, 105, 106]. Of course, FBW7 is responsible for the degradation of other proteins in the cell, among which include MYC [107, 108], and thus inactivation of FBW7 may support T-ALL pathogenesis in multiple, potentially synergistic ways. About 15% of T-ALL cases harbor both HD and PEST *NOTCH1* mutations in *cis* [78], and 5–10% harbor a *NOTCH1* HD mutation along with an *FBW7* mutation [79, 101, 102], both of which presumably lead to synergistic hyperactivation of NOTCH1 signaling.

Similar *Notch1* mutations involving the PEST domain also occur with high frequency in nearly all mouse models of T-ALL [109–115]. The paucity of spontaneous HD mutations in mouse models is presumably due to the prevalence of illegitimate RAG-dependent recombination within the mouse *Notch1* locus that deletes 5′ exons and results in expression of truncated peptides similar to those created by the t(7;9) in human disease [116]. Of note, irradiated SCID or $ATM^{-/-}$ mice also develop T-cell leukemias that also show frequent deletions in the proximal promoter and express similar N-terminally truncated NOTCH1 polypeptides [115]. These data reinforce the notion that there is strong selective pressure for activation of NOTCH1 signaling in T-cell transformation and support its prominent role in T-ALL pathogenesis, even in other organisms.

8.3.4 Clinical Significance of NOTCH1 Mutations in T-ALL

The presence of recurrent activating mutations in NOTCH1 raises the question whether these have any biologic or prognostic significance. Early studies showed that the activating mutations in *NOTCH1* correlated with improved clinical outcome, but subsequent studies suggest that this association is dependent on the therapeutic protocol [80, 83, 84]. More recent efforts to resolve this issue showed that *NOTCH1* and *FBW7* mutations were indeed associated with improved response to chemotherapy and in particular to glucocorticoids; however, this early benefit did not consistently translate into improvement in survival [117–119]. Moreover, *NOTCH1/FBW7* mutations were either not prognostic or possibly portended a worse outcome among high-risk patients. Of note, the association between activating *NOTCH1* mutations and improved response to glucocorticoid therapy did not affirm prior work that showed Notch inhibition with GSI could reverse glucocorticoid resistance [120], implying that such relationships are likely highly dependent upon genetic context.

8.3.5 *Genes and Pathways Downstream of NOTCH1 in T-ALL*

Several groups have contributed to defining the complement of genes and pathways which are ultimately activated downstream of NOTCH1 and that are functionally relevant to T-ALL pathogenesis (Fig. 8.3). Besides those already mentioned above, expression of *NOTCH3* is also induced by NOTCH1 in T-ALL [35, 38]. As there is substantial homology between ICN1 and ICN3, it is notable that ICN3 generates T-cell leukemia in mice similarly to ICN1 [70, 75, 121, 122]; however, deletion of *Notch3* has no effect on leukemia induction in the hypomorphic Ikaros-driven mouse T-ALL model, whereas deletion of *Rbpj* introduces a substantial delay [123], and spontaneous mutations in *NOTCH3* are conspicuously lacking in human T-ALL.

Activated NOTCH1 can potentiate PI3K/AKT/mTOR signaling by several means including repression of *PTEN* through HES1 [124] or upregulation of receptor tyrosine kinases (RTK) such as IL7 receptor (*IL7R*) [125, 126] and insulin-like growth factor 1 receptor (*IGF1R*) [127] (Fig. 8.3). Importantly, inhibiting PI3K/AKT/mTOR or upstream RTKs results in reduced growth and/or survival of T-ALL cells both in vitro and in vivo. Of note, PTEN loss, either by mutation [111, 124], silencing [128], or inactivation [129], can contribute to Notch-independent T-ALL cell growth by compensating for reduced Notch-dependent glutaminolysis with enhanced aerobic glycolysis [130]. This mechanism is indeed operative in many, but

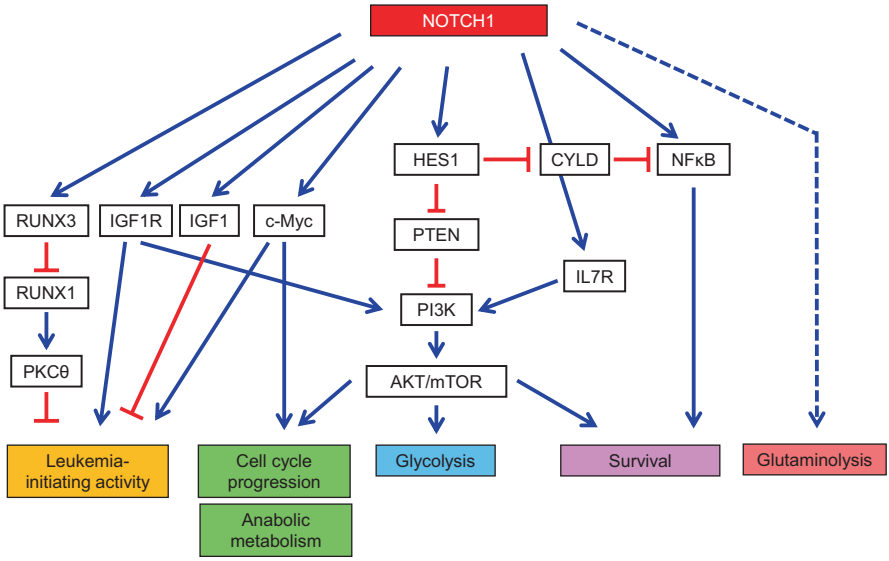

Fig. 8.3 Genes and pathways downstream of NOTCH1 in T-ALL. See text for details. RUNX3 runt-related transcription factor 3, RUNX1 runt-related transcription factor 1, PKCθ protein kinase C theta, IGF1R insulin-like growth factor 1 receptor, IGF1 insulin-like growth factor 1, c-Myc myelocytomatosis, HES1 hairy/enhancer of split 1, PTEN phosphatase and tensin homolog, PI3K phosphoinositide 3-kinase, mTOR mammalian target of rapamycin, CYLD cylindromatosis, NFκB nuclear factor kappa-light-chain-enhancer of activated B-cells, IL7R interleukin-7 receptor

not all contexts [131]. As well, the observation that PTEN loss accelerates NOTCH1-induced leukemogenesis [131] would support the notion that NOTCH1 and PI3K/AKT pathways function collaboratively and provide nonredundant contributions to T-ALL pathogenesis.

Other work has revealed interaction between NFκB and Notch pathways in T-ALL. Indeed, mouse T-cell leukemias, induced by activated NOTCH1 or NOTCH3, show high levels of NFκB activity [70, 132]. NOTCH1 induces NFκB activity directly by upregulating transcription of *Relb* and *Nfkb2* [133, 134], enhancing NFκB nuclear retention [135], and interacting physically with the IKK complex [134]. NOTCH1 also promotes NFκB activity indirectly by HES1-dependent repression of *CYLD*, a deubiquitinase that negatively regulates the IKK complex [136] (Fig. 8.3). Importantly, inhibition of NFκB activity antagonizes T-ALL cell growth/survival in vitro and in vivo [70, 134, 136].

8.3.6 NOTCH1 and Leukemia Stem Cells

Leukemia stem cells (and cancer stem cells more generally) have had a murky history, fraught with confusing and inconsistent use of terminology and misconceptions regarding what are core aspects of the concept versus what are related but non-requisite associations [137]. The term leukemia stem cells encompasses the overall concept that there is functional heterogeneity within a tumor whereby discrete subsets possess the unique ability to recreate the entire tumor in a naïve host. Accordingly, there must also be complementary subsets that are relatively devoid of such activity. This functional heterogeneity can be associated with but is not necessarily required to manifest as variation in phenotypic or morphologic differentiation. More specifically, leukemia stem cells need not express markers associated with hematopoietic stem cells, although they can as in the case of acute myeloid leukemia (AML) where they were first described [138]. Similarly, leukemia stem cells do not necessarily show evidence of existing in a less differentiated state than non-stem cells in the tumor population, owing mostly to the fact that the normal developmental sequence of marker acquisition in a given lineage is not necessarily preserved in transformed malignant cells. Finally, functional heterogeneity may coexist with genetic heterogeneity within a given tumor; however, the presence of the latter can potentially confound characterization of the former.

Incorporation of the term “stem” is meant to connote that they have the capacity to self-renew, similar to normal tissue stem cells. In the case of cancer, however, this property may either be retained at the initial point of cellular transformation or spontaneously acquired within a more differentiated cell by genetic alteration. Literally, this translates to the notion that leukemia stem cells are cancer cells that have stemlike properties and are not necessarily cancerous versions of normal tissue stem cells. As an experimental approach, serial transplantation (often performed at limiting dilution) into a naïve host is the gold standard for documenting that leukemia stem cells are indeed present within a given test population; however,

the assay itself actually measures so-called leukemia-initiating cell, or LIC, activity which is read out solely by the presence or absence of disease in the transplanted recipient [137].

In human T-ALL, several groups have demonstrated asymmetric localization of LIC activity within tumor subpopulations defined by surface markers including CD7, CD1a, and CD34 [139–141]. As well, LIC have been characterized within various mouse models of T-ALL [107, 142–147]. Signaling through NOTCH1 has been shown both in human and mouse T-ALL to sustain LIC activity [145, 148–150]. Work from our own group and others has identified *IGF1R*, *IGF1*, *PKCθ*, and *MYC* as relevant downstream targets of NOTCH1 that mediate its effects in supporting LIC [127, 149, 151] (Fig. 8.3). Implication of *MYC* has also prompted studies involving selective BET bromodomain inhibitors (e.g., JQ-1) that can potently silence *MYC* expression by epigenetic means and thus may represent a viable therapeutic strategy whose target range specifically includes LIC [107, 152]. Our work highlighting the transcriptional circuit linking NOTCH1 to repression of *PKCθ* and reactive oxygen species (ROS) via *RUNX3* and *RUNX1* also raises the possibility of targeting these other elements to specifically antagonize LIC [149].

8.4 Notch and B-Cells

8.4.1 Notch Signaling in Normal B-Cell Development

Although NOTCH1 signaling favors commitment of lymphoid progenitors to the T-cell lineage at the expense of B-cells, NOTCH2 has been shown to play a role later in B-cell development where it guides binary cell fate decisions between marginal zone (MZ) and follicular B-cells in the mouse spleen. Expression of *Notch2* increases with B-cell maturation, and deletion of either *Notch2* itself or *Rbpj* results in a complete failure of MZ B-cell development [153, 154]. This role of Notch signaling in MZ B-cell development was further confirmed in studies that knocked out other elements of the Notch signaling apparatus including MAML1 [155], DLL1 [57, 156], MIB1 [157], and ADAM10 [158], but interestingly does not require HES1 [159].

8.4.2 Notch Signaling in B-Cell Acute Lymphoblastic Leukemia (B-ALL)

Given the trophic effect of Notch activation on T-cell fate and leukemogenesis, yet suppressive role on early B-cell differentiation, it is notable that enforced expression of active forms of all four Notch receptors (ICN1-4) induced growth arrest and apoptosis in immature B-ALL cell lines which could be recapitulated by HES1

alone [3, 160]. Similar effects were seen in myeloma and Hodgkin cell lines. Subsequent work has shown that Notch/HES pathway elements are epigenetically silenced in B-ALL cell lines and patient samples as compared to T-ALL [161], supporting that signaling through Notch/HES is incompatible with the generation and/or maintenance of early B-cell malignancies.

8.4.3 *Notch Signaling in Chronic Lymphocytic Leukemia (CLL)*

CLL is a very common, low-grade malignancy of mature B-cells characterized by infrequently dividing but long-lived cells. The more aggressive form is thought to arise from naïve CD5+ B-cells with unmutated *IGHV* genes, whereas the less aggressive form shows *IGHV* mutations consistent with derivation from post-germinal center B-cells [162]. Early work showed NOTCH2 was responsible for driving expression of *CD23* [163], and aberrant activation of NOTCH1 and/or NOTCH2 supported survival/resistance to apoptosis via increased NFκB activity [164]. Mutations involving *NOTCH1* were first reported among 2 of 43 patients in 2009, and subsequent larger studies demonstrated *NOTCH1* mutations in about 10% of CLL cases at diagnosis, with higher incidence ~20% among patients with chemorefractory disease and ~30% in cases that had progressed/undergone Richter transformation [89–91]. While *NOTCH1* mutations in T-ALL target both HD and PEST domains, mutations in CLL are restricted to the PEST domain, and strikingly, over 80% of these are represented by the exact same 2bp deletion (ΔCT75447545, P2515fs) that results in frameshift and premature stop codon to delete the PEST degron. With more sensitive, allele-specific PCR-based methodologies, the *NOTCH1* c.7544_7545delCT PEST mutational frequency has been reported as high as 20% among unselected patients [93] and even higher at ~74% among trisomy 21 patients (C. Hoofd et al., manuscript in preparation) [165, 166]. Though *NOTCH1* PEST mutations are associated with unmutated *IGHV* genes and wild-type *TP53*, they represent an unfavorable prognostic factor independent of both *IGHV* and *TP53* status [91, 167, 168]. Mutations within the noncoding region of NOTCH1 have also been reported to occur in CLL that cause aberrant splicing and result in expression of truncated forms lacking the C-terminal PEST domain [169].

Immunohistochemical studies that are able to detect activated ICN1 in the nucleus of CLL cells have revealed the pathway to be activated in nearly 90% cases, occurring similarly in *NOTCH1* mutated and non-mutated groups [170, 171]. NOTCH1 activation is lost rapidly in vitro, irrespective of mutational status [172], suggesting that signaling relies upon stroma-derived ligand within the tumor microenvironment.

8.4.4 Notch Signaling in Mantle Cell Lymphoma (MCL)

Mantle cell lymphoma (MCL) is a less common but more aggressive type of mature B-cell non-Hodgkin lymphoma that is molecularly defined by the t(11;14)(q13;q32) chromosomal translocation which results in overexpression of cyclin D1 (CCND1) [173]. Our group identified gain-of-function *NOTCH1* mutations, nearly exclusively by PEST deletion, to occur in 12% of MCL cases ($n = 108$) and to be associated with poor prognosis [94]. Half of these were represented by the c.7544_7545delCT mutation seen in CLL. A subsequent study found *NOTCH1* and *NOTCH2* mutations each to occur at ~5% among a cohort of 172 MCL cases, were restricted to the PEST domain, and tended not to co-occur within the same tumor [95]. *NOTCH1/2* mutations in this cohort were also associated with poor clinical outcome. In contrast to CLL, immunohistochemical staining for ICN1 failed to reveal evidence for widespread activation of NOTCH1 in MCL tissues [170, 171].

8.4.5 Notch Signaling in Splenic Marginal Zone Lymphoma (SMZL)

Splenic marginal zone lymphoma (SMZL) is another uncommon but indolent mature B-cell non-Hodgkin lymphoma with recurrent chromosome 7q deletions [174] and activation of the NFκB pathway [175]. SMZL has been associated with hepatitis C virus (HCV) infection [176], and interestingly, some patients show responses to antiviral therapy [177]. The role of NOTCH2 in MZ B-cell development in mice perhaps portended the finding by two groups of recurrent *NOTCH2* mutations in ~20–25% of SMZL cases, again with a preponderance resulting in deletion of the C-terminal PEST domain, but a rare activating HD mutation was also observed [96, 97]. The clinical significance of *NOTCH2* mutations in SMZL remains unclear, however, as the two studies reported opposite results for their respective patient cohorts (longer overall survival, $n = 94$ vs. shorter relapse-free survival, $n = 46$). Of note, one of the studies also identified the *NOTCH1* c.7544_7545delCT mutation to occur at ~5% within their SMZL cohort [96, 97]. As in MCL, identified *NOTCH1* and *NOTCH2* mutations in SMZL were mutually exclusive.

8.4.6 Notch Signaling in Other Non-Hodgkin Lymphomas

Follicular lymphoma (FL) is one of the most common nodal non-Hodgkin lymphomas, second only to diffuse large B-cell lymphoma (DLBCL). While many cases of FL are indolent and slow-growing, approximately 2–3% of FL patients per year will

undergo histologic transformation to a more aggressive lymphoma, often DLBCL [178]. As reported in abstract form, mutations in *NOTCH1* and *NOTCH2* were identified in FL to occur at a combined frequency of ~6% (five mutations in *NOTCH1* and two mutations in *NOTCH2* among a cohort of 114 FL cases) [179]. These mutations were all predicted to encode truncated proteins lacking the C-terminal PEST domain. Formal publication of this study, however, remains pending.

Recurrent mutations in *NOTCH2* were reported to occur in ~8% of DLBCL cases ($n = 63$) and were represented mostly as causing deletion of the PEST domain [98]. Mutations in both *NOTCH1* and *NOTCH2* have also been identified in DLBCL associated with HCV infection, occurring at frequencies of 4% and 26%, respectively, among of cohort of 46 cases [180]. These mutations were also exclusively of the PEST deletion variety and were associated with poor clinical outcome in this small cohort. Given the association between HCV and SMZL and the similarities in *NOTCH1/2* mutation frequency and pattern with that observed in SMZL, it remains possible that these cases of HCV-associated DLBCL may have arisen by transformation from a preexistent but unrecognized SMZL clone.

Multiple myeloma (MM) is a malignancy of terminally differentiated plasma cells that typically affects older adults. While recurrent gene mutations affecting the Notch pathway have not been reported in this disease, several studies have shown upregulation of Notch receptors and/or ligands including NOTCH1, NOTCH2, JAG1, and JAG2 [181, 182]. Moreover, pharmacologic inhibition of Notch signaling has been shown to prevent localization of MM cells to the bone marrow [183] and enhance their sensitivity to chemotherapy [184].

8.4.7 Notch Signaling in Classical Hodgkin Lymphoma

Classical Hodgkin lymphoma is characterized by a relatively minor proportion of malignant Hodgkin and Reed-Sternberg (HRS) cells that are thought to derive from “crippled” germinal center B-cells [185] and which secrete abundant cytokines, resulting in the bulk of the tumor mass being composed of infiltrating reactive immune cells. Immunohistochemical studies have revealed that HRS cells within patient tumors express both NOTCH1 and JAG1 highly, and that nearby stromal cells also express JAG1 [184, 186, 187], suggesting that ligand-dependent activation of Notch signaling in HRS cells may occur by homo- and heterotypic cell interactions. Cultured HRS cell lines express both NOTCH1 and NOTCH2 and respond to JAG1 ligand with increased proliferation and reduced apoptosis. Additional cell line studies from the same group have suggested that Notch signaling supports cell survival through activation of the alternative NFκB pathway [188].

8.5 Notch and Myeloid Cells

8.5.1 Notch Signaling in Normal Myeloid Development

The role of Notch signaling in myeloid development is ambiguous as several reports have suggested that Notch activation may alternately promote or inhibit various aspects of granulocyte/monocyte differentiation [189–192], yet conditional knockout of *Rbpj* and enforced expression of dominant-negative MAML1 both showed no impairment of myeloid lineage commitment or differentiation [49, 51]. As well, there are conflicting reports that Notch signaling either promotes or antagonizes megakaryocyte differentiation [193, 194]. Taken together, these studies suggest that Notch plays a complex role in myeloid cell fate decisions that will require further study to resolve.

8.5.2 Notch Signaling in Myeloid Leukemia

Early studies have found that despite high expression of NOTCH1 receptors in acute myelogenous leukemia (AML) patients, activation of the pathway was limited [195, 196]. Moreover, exposure to DLL1 and JAG1 ligands produced variable outcomes in terms of short-term growth of primary patient AML blasts [197] which echoed prior findings with established AML cell lines [198, 199]. More recently, a pair of studies examined gene expression profile data from large cohorts of AML patients and confirmed the expression of multiple Notch receptors; however, pathway activation was again found to be limited compared to normal hematopoietic cells [200, 201]. Interestingly, enforced expression of activated NOTCH1 (ICN1) blocked proliferation and induced apoptosis in AML cell lines and patient samples and antagonized LIC activity in an MLL-AF9-induced mouse model of AML. As well, enforced expression of HES1, a transcriptional repressor immediately downstream of Notch, led to growth arrest both in vitro and in a xenograft mouse model [200, 202], an effect that may be mediated through repression of *FLT3* [203].

Mice doubly deleted for *Notch1/Notch2* or just nicastrin (*Ncstn*), a component of the γ-secretase complex responsible for activation of Notch receptors, leads to the development of a myeloproliferative disorder in mice [204]. Additional studies showed that loss of nicastrin in multipotent hematopoietic progenitors was associated with induction of a broad myeloid transcriptional program, an effect that was reversed in part by enforced expression of HES1. These findings led the investigators to search for evidence of loss of Notch signaling function in human myeloproliferative disorders, and indeed they found 6 somatic loss-of-function mutations involving *NCSTN*, *APH1*, *MAML1*, and *NOTCH2* within 5 of 42 samples (12%) from patients with chronic myelomonocytic leukemia (CMML). Taken together, these studies support the notion that Notch signaling may act as a tumor suppressor

in the myeloid cell context and that therapies that activate Notch signaling may have clinical utility in myeloproliferative disease.

8.6 Therapeutic Approaches to Target the Notch Pathway

The relevance of Notch signaling in T-ALL and other hematologic malignancies has created interest in the development of various pharmacologic modulators of the pathway. The first volley of agents were small molecule inhibitors of γ-secretase, which is required for proteolytic cleavage of all four Notch receptors and liberation of their respective ICN subunits from the plasma membrane. γ-Secretase inhibitors, or GSIs, were ripe for plucking as these drugs were already in clinical development to prevent processing and accumulation of β-amyloid from amyloid precursor protein (APP), a candidate etiology in Alzheimer's disease progression [205]. The antitumoral activity of several GSIs (e.g., MRK-003, MRK-0752, and RO4929097) has been already tested in mouse models of T-ALL in phase I clinical trials for patients with relapsed T-ALL [206–209]. These efforts were stymied, however, by dose-limiting toxicities primarily affecting the gut where pan-Notch inhibition leads to goblet cell hyperplasia and resultant severe diarrhea. Subsequent work revealed that this effect required inhibition of both NOTCH1 and NOTCH2 in intestinal crypt progenitors [210, 211] but could be ameliorated either by intermittent dosing [208] or rescue with glucocorticoids [120].

Another strategy proposed the use of chemically stapled α-helical peptides [212] similar to dnMAML1 [76, 213] to render the Notch transcriptional complex functionally inert; however, this approach has remained in the research literature thus far.

Antibodies have also been designed against specific regions of Notch receptors, specifically the negative regulatory region (NRR), on the premise that these would help to stabilize the receptor heterodimer and restrict ADAM protease-mediated receptor cleavage and activation, induced either by ligand binding or mutations involving the HD domain [77, 214–217]. One issue that has arisen, however, is lower activity of NRR-directed antibodies as compared to GSI, possibly due to incomplete allosteric inhibition of the ligand-induced conformational change [214]. Other targets for therapeutic antibodies include the ligand-binding EGF repeats of Notch receptors, or alternatively, the ligands themselves [218, 219]. Identification of additional targets, including those effectors downstream of Notch signaling that contribute ultimately to enacting cellular phenotypes, as well as further development of specific pharmacologic agents will be required to capitalize upon our knowledge of the role Notch signaling plays in human disease.

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